The structure of a pyoverdine from *Pseudomonas* sp. CFML 96.188 and its relation to other pyoverdines with a cyclic C-terminus

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Abstract

From *Pseudomonas* sp. CFML 96.188 a pyoverdine was isolated and its primary structure was elucidated by spectroscopic methods and degradation reactions. This strain is of interest as it accepts the structurally different pyoverdines from several other *Pseudomonas* strains. They all have in common as a specific structural feature a C-terminal cyclic substructure, the importance of which for the recognition of a pyoverdine at the cell surface of a given strain will be discussed.

Abbreviations: Common amino acids, 3-letter code; Ac(Fo)OHOrn–δ-N-acetyl(formyl)-N-hydroxy Orn; cOHOrn–cyclo-N-hydroxy Orn; Kgl–2-ketoglutaric acid residue; Suc–succinic acid residue; Suca-succinamide residue; Chr–pyoverdine chromophore (see Figure 1); TAP–N/O-trifluoroacetyl (amino acid) isopropyl ester; RP-HPLC–reversed phase high performance liquid chromatography; GC–gas chromatography; ESI–electrospray ionization; FAB–fast atom bombardment; MS–mass spectrometry; NMR–nuclear magnetic resonance; COSY–correlated spectroscopy; DEPT–distortionless enhancement by polarization transfer; HMBC–heteronuclear multiple bond correlation; HMQC–heteronuclear multiple quantum coherence; ROESY-rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY–total correlation spectroscopy; WATERGATE–water suppression by gradient-tailored excitation; DSS–2,2-dimethyl-2-silapentane-5-sulfonate; TMS–tetramethylsilane; IEF–isoelectrofocusing; CFML–Collection de la Faculté de Médecine de Lille.

Introduction

Though the existence of the pseudomonad fluorescent pigments had been known for almost 130 years (for a historical survey see Voßen *et al.* 2000) a systematic investigation did not start before the structure of the first representative had been established (Teintze *et al.* 1981). Today over 40 complete or fairly complete structures of pyoverdines were elucidated (Kilz *et al.* 1999) and from preliminary studies it appears that many more are to be expected (Meyer 2000). Pyoverdines, which have been demonstrated to act as powerful Fe^{3+}-scavengers and siderophores for their producing strains, consist of three distinct structural parts, viz. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain comprising 6 to 12 amino acids bound to its carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amidically to the NH_2-group (cf. I, Figure 1) (Budzikiewicz 1997a, b). In most cases, the variability of the peptide chain safeguards that a given ferri-pyoverdine is available only to the producing strain because of a highly specific interaction between the pyoverdine and its receptor outer membrane protein (Hohnadel & Meyer 1988). There are, however, examples known where *Pseudomonas* spp. can accept ferri-

pyoverdines differing in the peptide chain from the one produced by themselves, as certain *P. fluorescens* and *P. putida* strains (Jacques *et al.* 1995; Georgias *et al.* 1999) or *P. aeruginosa* ATCC 15692 which recognizes the ferri-pyoverdine of *P. fluorescens* ATCC 13525 (Hohnadel & Meyer 1988; Kinzel *et al.* 1998). It is still an open question whether in such cases the foreign ferri-pyoverdine is accepted by the receptor of the own one due to structural similarities (Meyer *et al.* 1999), or whether a new receptor is developed (Koster *et al.* 1993). *Pseudomonas* sp. CFML 96.188 is of special interest in this respect as it accepts the ferri-pyoverdines of six other strains differing in their structure but having in common a C-terminal cyclic substructure. It will be shown that the structure of the pyoverdine from *Pseudomonas* sp. CFML 96.188 fits into this scheme.

Materials and methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT H-SQ 30 (FAB, matrix thioglycerol/dithiodiethanol), Finnigan-MAT 900 ST (ESI); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: DPX 300 (^{1}H 300, ^{13}C 75.5 MHz) and DRX 500 (^{1}H 500, ^{13}C 125 MHz) (both Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ(TMS) = δ(DSS) for ^{1}H, δ(DSS) = -1.61 ppm for ^{13}C. Suppression of the H_2O signal by the WATERGATE puls sequence.

UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen), CD: Jasco J-715 (Jasco, Tokyo, Japan).

Chromatography: RP-HPLC columns Nucleosil 100-C_{18} (5 μm) and Eurospher 100-C_{18} (7 μm) (Knauer, Berlin); low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), CM-Sephadex C-25 (Pharmacia, Uppsala, S), Sep-Pak RP$_{18}$ cartridges (Waters, Milford MA, USA); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt); thin layer chromatography: Mikropolyamide F1700 (Schleicher & Schüll, Dassel).

Chemicals: Water was desalted and distilled twice in a quartz apparatus; for HPLC it was further purified on XAD-4 resin and filtered through a sterile filter. Organic solvents were distilled over a column. Reagents were of p. a. quality.

Production and isolation of the pyoverdines

Pseudomonas sp. CFML 96.188 isolated from spring water in Lebanon (Dabboussi *et al.* 1998) was grown in a succinate minimal medium (Budzikiewicz *et al.* 1997). For the work-up of the culture and isolation of the ferri-pyoverdines by chromatography on XAD-4 and Biogel P-2 see Georgias *et al.* (1999). Two fractions were obtained (Ia and Ib) which were further purified by chromatography (twice) on CM-Sephadex C-25 with a 0.1 M pyridinium acetate buffer (pH 5.0). Purity was checked by RP-HPLC on Nucleosil C_{18} with a 0.1 M ammonium acetate buffer (pH 6.2)/methanol (gradient 3 to 78% buffer). Decomplexation was achieved by adsorption of the ferri-pyoverdines on a Sep-Pak cartridge and washing with a K oxalate buffer (pH 4.0). After removing excess oxalate with an 1 N aqueous solution of NaCl the free pyoverdines were eluted with methanol/water 1:1 (v/v). The solutions were brought to dryness i.v. and the residues was stored at -25 °C.

For qualitative and quantitative analysis of the amino acids obtained by total hydrolysis, determination of their configuration by GC/MS of their TAP derivatives on a chiral column and dansyl derivatization of free amino groups see Briskot *et al.* (1986) and Mohn *et al.* (1990). Partial hydrolysis of Ib was achieved with 6 N HCl at 90 °C for 10 min. The peptide fragments were separated and purified by chromatography on Bio-Gel P-2 (three times) with 0.1 M acetic acid. Subsequently they were subjected to total hydrolysis, TAP derivatization and GC-analysis as above.

The Ga complex of Ib was prepared by addition of a 1.5 molar excess of Ga(NO_3)$_3$ to a solution of the pyoverdine in a 0.2 M pyridinium acetate buffer solution (pH 5.0). After 1 h the reaction mixture was brought to dryness i. v., redissolved several times in water and brought to dryness again to remove all pyridinium acetate. The residue was dissolved in water, the Ga complex was adsorbed on a Sep-Pak cartridge, washed with water and desorbed with methanol/water 1:1 (v/v); the eluate was brought to dryness, its purity was checked with RP-HPLC on Nucleosil-C_{18}.

For oxidative decarboxylation (cf. Briskot *et al.* 1986) to a solution of 10 mg of ferri-Ia in 10 ml 0.01 M phosphate buffer (pH 8.0) 5 ml 30% H_2O_2 were added. After standing for 4 h at room temperature the thus obtained ferri-Ic was adsorbed on an activated Sep-Pak cartridge, washed with 10 ml H_2O and eluted with CH_3OH/H_2O 1:1 (v/v). The solution was brought to

Figure 1. Structure of pyoverdine I.

Ia: R = CH$_2$-CH$_2$-CO-COOH
Ib: R = CH$_2$-CH$_2$-CHNH$_2$-COOH
Ic: R = CH$_2$-CH$_2$-COOH
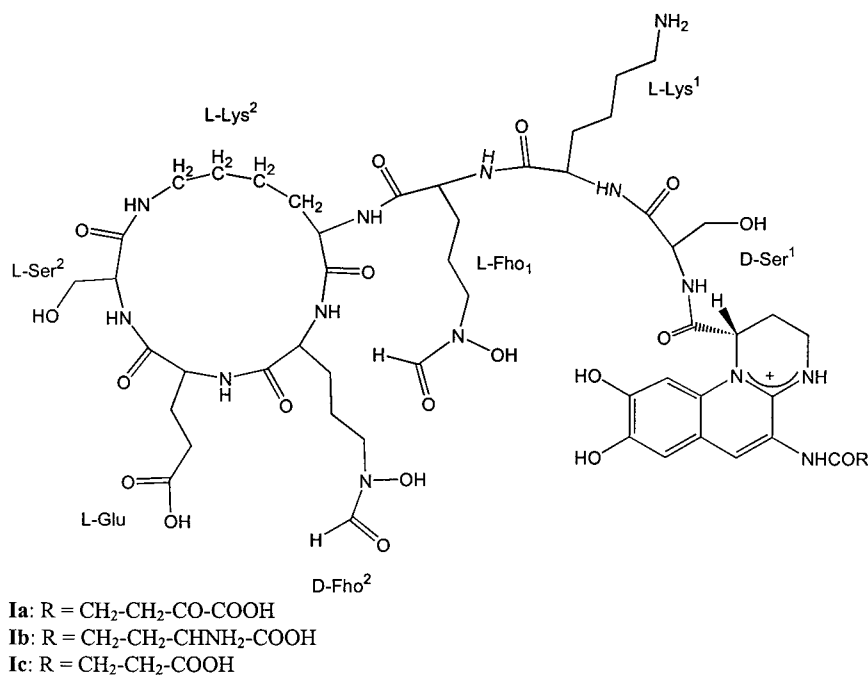
dryness i.v. and Ic was purified by chromatography on CM-sephadex C-25 with 0.1 M pyridinium acetate buffer (pH 5.0).

Uptake studies and isoelectrofocusing (IEF)

The methods previously described for uptake studies with ^{59}Fe labelled pyoverdines (Munsch *et al.* 2000) and for pyoverdine isoelectrofocusing (PVD-IEF, Meyer *et al.* 1998) were used. The values given in Figure 3 were measured after 20 min of incubation and corrected for blank values obtained in essays without bacteria. 100% incorporation corresponded to 20,800 cpm of the 50,000 cpm available in the assay. The isoelectric pH values (pI) depend on the presence of basic and acidic groups present in the pyoverdine. Moreover, the co-occurance in the culture supernatant of several pyoverdine forms (isoforms) with an identical peptide chain, but different side chains (succinic, ketoglutaric acid, Glu etc.) results in several bands during isoelectrophoresis, thus conferring to a given strain a characteristic pyoverdine-IEF pattern.

Results

Characterization of Ia - Ic

The UV/Vis spectrum of Ia is characteristic for pyoverdines, especially the pH dependance for the free siderophore and the broad charge-transfer bands of the Fe-complexes (Budzikiewicz 1997a, b). The molecular masses of Ia - Ic were determined by FAB- and ESI-MS as 1261, 1262 and 1233u. The *retro*-Diels-Alder fragments (Michels *et al.* 1991) at m/z 930 corresponding to M - 331, 332 and 303, respectively (loss of the quinoline ring together with the side chain) confirm the presence of the side chains Kgl (Ia), Glu (Ib) and Suc (Ic).

Gas chromatographic analysis of the TAP derivatives after total hydrolysis of Ia and Ib gave L-Glu, L-Lys, D- and L-Orn, D- and L-Ser; the abundance of L-Glu is higher for Ib than for Ia in agreement with the presence of a Glu side chain in addition to the Glu in the peptide chain (Kgl due to its decomposition under the conditions of hydrolysis can not be detected in the hydrolysate; its presence in Ia was, however confirmed by MS - see above, NMR - see below - and by oxidative degradation to succinic acid, Ic).

By total hydrolysis after dansylation only ε-dansyl Lys was obtained from Ia as could be shown by chromatograhic comparison with samples of authentic α-

and ε-dansyl Lys. Hence in Ia at least the ε-amino group of one Lys, but in neither case an α-amino group is free. As no α-dansyl Ser was detected Ser must be incorporated into the peptide chain also by its α-amino group.

Determination of the amino acid sequence

Basis for the sequence determination by NMR is the unambiguous identification of all ^{1}H-and ^{13}C-signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: H,H-COSY allows to detect the ^3J-, TOCSY higher H,H-couplings within one amino acid residue (amide bonds interrupt the scalar H,H-coupling). HMQC identifies ^1J-C,H, HMBC ^2J- and ^3J-coupling, and allows thus to identify also quaternary C-atoms. DEPT distinguishes between CH/CH$_3$ and CH$_2$-signals, while ^{13}C-signals of quaternary C-atoms are missing. Sequence information is obtained by ROESY which correlates NH-protons (sharp signals are obtained with ca. 15 mM solutions in an aqueous phosphate buffer pH 4.3, suppression of the H$_2$O signal by presaturation or by the WATERGATE method) with spatially close α- and β-H's of the preceding amino acid (**CH**-**CH**-CO-N**H**) and by HMBC correlating amide-CO with the α-H of the following amino acid (see Figure 2). The ^{1}H- and ^{13}C-data of Ib are compiled in Tables 1 and 2. They correspond to those observed with other pyoverdines (Budzikiewicz 1997a, b).

The following signals deserve a comment: the NH-signal of Ser^1 bound directly to the carboxyl group of the chromophore is typically shifted downfield. The shift values of the CH$_2$-groups of Ser (3.98 and 3.89 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected) (Budzikiewicz 1997b). For the two FoOHOrn residues the signals for the *cis/trans*-isomers can be seen. The incorporation of Lys by its α- and/or ε-amino group into the peptide chain had been discussed in detail by Budzikiewicz *et al.* (1999) (cf. also Sultana *et al.* 2000). The dansylation experiment showed that at least one Lys has a free ε-amino group. The shift values for Lys^1 fall into the range given there for Lys bound peptidically by its α-amino group into the peptide chain, but for cyclic structures (Lys^2) wider ranges have to be expected.

The C-^{1}H and -^{13}C shifts of Ia and Ic differ from those of Ib by less than 0.1 ppm (^{1}H) and less than 1 ppm (^{13}C), respectively, with the exception of the chromophore signal CH-6 of Ia: The ketoglutaric acid side chain in aqueous solution cyclizes by attack of the NH- at the α-CO group (Briskot *et al.* 1986; for the equilibrium structures see Sultana *et al.* 2000), hence instead of a CO signal those of quaternary C-atoms at 94.4 and 95.5 can be seen due to the two possible stereoisomers (-N-<u>C</u>(OH, COOH)-CH$_2$-). The formation of the cyclic structures somewhat influences the shift value of C-6 of the chromophore (142.4 ppm). The shift values of the Glu side chain confirm the attachment to the C-4 NH group of the chromophore by the γ-carboxyl group (see Budzikiewicz 1997b). Neighboring the amide NH signals additional signals of low intensity can be observed (slightly more abundant at 278K as compared with 303K, at higher temperatures decomposition of the sample commences) which can be explained by the presence of a second conformer (Cf. the discussion of the Ga complex below). This phenomenon was observed before (Barelmann 1999).

The peptide sequence as derived from ROESY and HMBC correlations is given in Figure 2. The molecular masses of Ia - Ic correspond to the structural details discussed above (amino acids, one cyclic substructure, Kgl/Glu/Suc side chain).

Location of the stereoisomeric amino acids (Ser, Orn)

Three fractions of the partial hydrolysate of Ib were isolated by chromatography containing (a) Chr-Ser^1-Lys^1, (b) Glu-Chr-Ser^1-Lys^1 together with the same sequence minus H$_2$O, and (c) Glu-Chr-Ser^1-Lys^1-OHOrn^1 together with the same sequence minus H$_2$O. Total hydrolysis, TAP derivatization and GC analysis on a chiral column gave D-Ser and L-Lys for (a), D-Ser, L-Glu and L-Lys for (b), and D-Ser, L-Glu, L-Orn and L-Lys for (c). From these findings the configuration of the first three amino acids follows as D-Ser-L-Lys-L-Orn.

NMR investigation of the Ga^{3+} complex

The paramagnetism of Fe^{3+} makes an NMR study of Fe-pyoverdines impossible. However, Ga^{3+} complexes can be used as a model; the ion radius of Ga^{3+} (62 pm) hardly differs from that of Fe^{3+} (64 pm). The formation of a 1:1 complex of Ib was confirmed by its molecular mass as determined by ESI-MS (M+Ga-3H), by the pH-independent absorption at 400 nm and the green fluorescence upon irradiation at 366 nm (Barelmann 1999).

NMR-data allow to identify the complexing sites of the metal ion and offer some information regard-
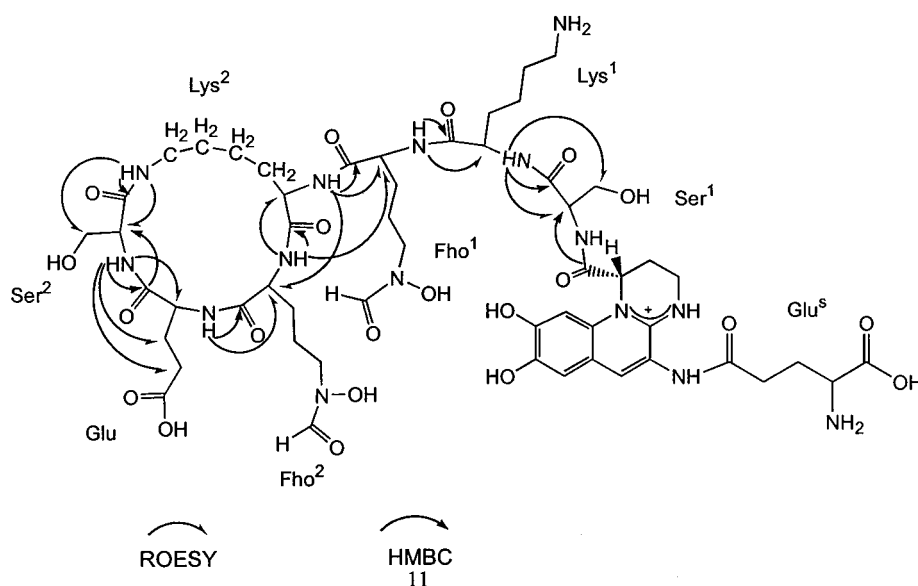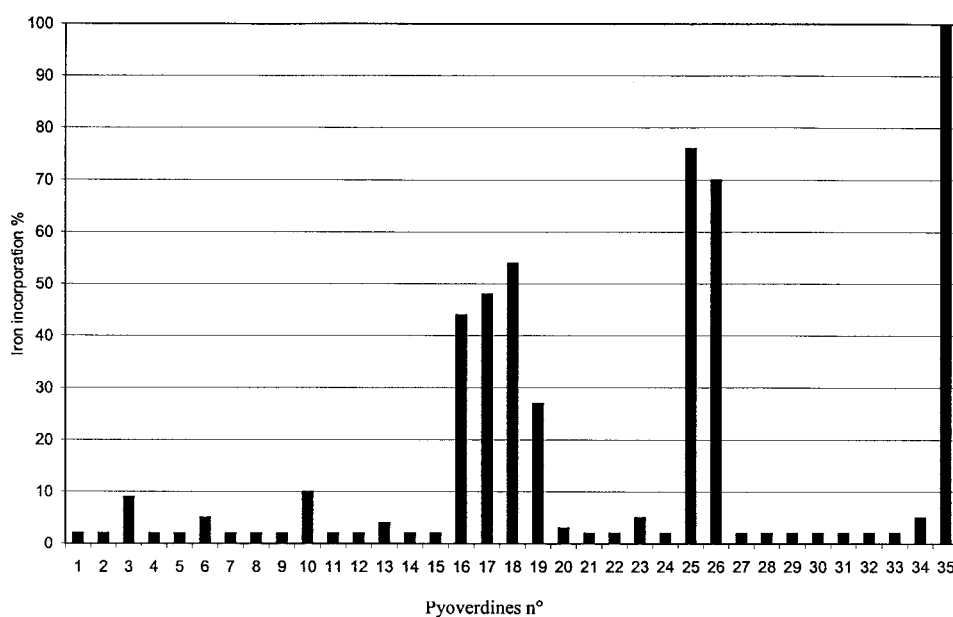
Figure 2. NMR correlations for pyoverdine Ib.

Figure 3. Heterologous pyoverdine-mediated $^{59}Fe^{3+}$-incorporation in *Pseudomonas* sp. CFML 96-188 given as percentage of incorporation reached with the homologous pyoverdine I.

The pyoverdines tested originated from the following *Pseudomonas* strains: 1. P.sp. strain E8; 2. *P. syringae* ATCC 19310; 3. *P. fluorescens* 9AW; 4. *P. putida* ATCC 12633; 5. *P. fluorescens* 51W; 6. *P. aeruginosa* Pa6; 7. *P. fluorescens* CCM 2798; 8. *P. fluorescens* CHA0; 9. *P. tolaasii* NCPPB 2192; 10. *P. aeruginosa* ATCC 27853; 11. *P. fluorescens* ii, 12. *P. fluorescens* SB8.3; 13. *P. fluorescens* ATCC 17400; 14. *P. fluorescens* 1.3; 15. *P.* sp. strain 267; 16. *P. fluorescens* ATCC 13525; 17. *P. aeruginosa* ATCC 15692; 18. *P. fluorescens* 18.1; 19. *P. fluorescens* 12; 20. *P. fluorescens* CFBP 2392; 21. *P. putida* CFBP 2461; 22. *P.* sp. ATCC 15915; 23. *P. monteilii* CFML 90-54; 24. P. 'mosselii' CFML 90-77; 25. *P. rhodesiae* CFML 92-104; 26. *P. veronii* CFML 92-124; 27. *P.* sp. CFML 90-40; 28. *P.* sp. CFML 90-42; 29. *P.* sp. CFML 90-51; 30. *P.* sp. CFBP 4396; 31. *P.* sp. strain 7SR1; 32. *P.* sp. strain 2908; 33. *P.* sp. A214; 34. *P.* sp. strain Ps3a; 35. *P.* sp. CFML 96-188 (homologous pyoverdine I).

Table 1. ^{1}H-NMR data of Ib (H_2O/D_2O 9:1, pH 4.3, 25 °C), correlations by TOCSY and NOESY

Glu	CH$_2$	CH$_2$	CHNH$_2$
	2.76	2.23	3.84

Chr	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.72	2.50	2.69	3.40	3.74	*	7.91	7.13	7.12	*

Amino acid	α-NH	α	β	γ	δ	ε	ε-NH$_2$	CHO$_Z$	CHO$_E$
Glu	9.08	4.11	2.04	2.35					
Lys^1	8.63	4.29	1.68	1.25	1.55	2.82	*		
			1.86						
Lys^2	7.72	3.84	1.61	1.19	144	2.88	7.39		
			1.73		1.55	3.56			
Ser^1	9.55	4.37	3.97						
Ser^2	8.30	4.47	3.89						
FoOHOrn^1	8.14	4.06	1.31	1.60	3.42$_Z$			7.90	8.28
			1.59		3.46$_E$				
FoOHOrn^2	7.59	4.43	1.71	1.72^a	3.53$_Z$			7.91	8.26
					3.58$_E$				

*Not observed.

ing the three-dimensional structure of the complex. Expectedly the ^{13}C resonances of the carbon atoms whose oxygen functions are engaged in the complexation show considerable differences from those of the uncomplexed ligand: The catecholate system (C-8 and C-9 of the chromophore) suffers a downfield shift of 6.9 and 7.4 ppm, respectively, and the formyl frequences of FoOHOrn^1 and FoOHOrn^2 are shifted upfield by 6.1 and 6.8 ppm, respectively. In the ^{1}H spectrum considerable upfield shift differences are observed for Lys^1 suggesting its location above or below the aromatic system, while the downfield shift of the CH protons of Orn^1 and of the NH protons of Lys^2 and of Orn^2 places them in the plane of the aromatic rings. The additional signals of the NH protons observed for the free Ib have disappeared. Apparently only one steric configuration prevails.

Additional siderophores and related compounds

The main siderophores produced by the strain under investigation are the pyoverdines Ia and Ib differing in the nature of the chromophore side chain (Kgl and Glu). An LC-ESI-MS coupled with an UV/Vis analysis of the crude XAD extract (Kilz *et al.* 1999) reveals the presence of minor components of the fermentation broth, pyoverdines with other side chains (here with malic acid) and precursors (Böckmann *et al.* 1997), viz. a 5,6-dihydropyoverdine with a Kgl side chain, and of 5,6-dihydro-7-sulfonic acids with Kgl, Glu and Suca side chains.

Siderotyping studies

Pseudomonas sp. CFML 96.188 grown in a succinate medium was tested for its capacity to incorporate [59]Fe^{3+} as mediated by its own pyoverdine (I, n° 35 in Figure 3) and by a collection of 34 structurally different pyoverdines listed in the legend of Figure 3. The structures of most of them are known (Budzikiewicz 1997a; Kilz *et al.* 1999), some are still under investigation. Six of the pyoverdines tested (no 16–19, 25 and 26) were accepted by *Pseudomonas* 96.188, but the incorporation rate was lower than for the homologous pyoverdine. For all the other pyoverdines no or less than 10% incorporation was observed. I and the six accepted pyoverdines show distinct IEF patterns (Table 3) in accordance with their different peptide chains. The structures of n° 16–19 are known (Hohlneicher *et al.* 1995; Briskot *et al.* 1986; Amann *et al.* 2000; Geisen *et al.* 1992) and for n° 25 the amino acid sequence (without D,L-assignments) was suggested from MS analysis (Fuchs 2000). N° 26 (*P. veronii*) presents a problem: according to the MS analysis its peptide chain should be identical with that of n° 16, but the IEF data (Table 3) and the uptake rate (Figure 3) of these two pyoverdines differ somewhat. As the isoforms of a pyoverdine reflect the nature of the acid side chains, change in their pattern could explain the shift of one band. However, the acid side chain should not influence the uptake rate. Provided the two pyoverdines do possess the same peptide chain

Table 2. ^{13}C-NMR data (δ [ppm]) of Ib (H$_2$O/D$_2$O 9:1, pH 4.3; 25 °C)[a]

Glu	CO	CH$_2$	CH$_2$	CHNH$_2$	COOH		
	176.6	32.5	26.7	55.2	175.2		

Chr	CO	1	2	3	4a	5	6
	171.6	57.7	22.9	36.3	150.2	118.5	139.6
	6a	7	8	9	10	10a	
	115.5	114.8	145.2	153.6	101.2	132.6	

Amino acid	CO	α	β	γ	δ	ε	CHO$_Z$	CHO$_E$
Glu	175.7	57.2	28.0	34.5	181.9			
Lys[1]	175.4	54.9	30.8	23.2	27.2	40.4		
Lys[2]	175.5	56.6	30.6	21.4	27.9	39.4		
Ser[1]	173.6	58.4	61.8					
Ser[2]	172.3	56.5	61.8					
FoOHOrn[1]	174.6	54.9	27.8	23.9	50.7$_Z$		160.5	164.8
					46.7$_E$			
FoHOOrn[2]	176.3	53.8	29.2	24.2	60.0$_Z$		160.5	164.8
					47.0$_E$			

[a]Based on HMBC and HSQC spectra.

Table 3. Structures of pyoverdines with C-terminal cyclopeptidic rings[a] and their pI values; the numbers correspond to those in Figure 3

Pyover dines			Amino acids						pI
35	<u>Ser</u>	Lys		Fho				(Lys-<u>Fho</u>-Glu-Ser)	7.4 - 5.3 - 4.3
25	<u>Ser</u>	Lys		Fho	Ser			(Lys-Fho-Ser-Ser)[b]	8.2 - 8.0 - 7.1 - 7.0
19	<u>Ser</u>	Lys	Gly	Fho	<u>Ser</u>	Ser	Gly	(Lys-<u>Fho</u>-Glu-Ser)	7.1 - 4.9 - 4.8
18	<u>Ser</u>	Lys	Gly	Fho	Ser	<u>Ser</u>	Gly	(Lys-<u>Fho</u>- -Ser)	8.5 - 7.4 - 7.1
16	<u>Ser</u>	Lys	Gly	Fho				(Lys-<u>Fho</u>- -Ser)	8.7 - 7.3 - 7.1
26	Ser	Lys	Gly	Fho				(Lys-Fho- -Ser)[b]	8.5 - 7.3 - 7.1
17	<u>Ser</u>	Arg	<u>Ser</u>	Fho				(Lys-Fho-Thr-Thr)	8.8 - 7.0

[a]D-Amino acids are underlined; Fho = FoOHOrn; [b]Amino acid sequence determined by MS, stereochemistry of the amino acids not known.

an explanation could for instance be a different rate of ferribactin/pyoverdine production (ferribactins considered to be precursors of pyoverdines - Böckmann *et al.* 1997 - and seem not to act as siderophores).

Discussion

Pseudomonas CFML 96.188 by its main growth characteristics should belong to the classical *P. fluorescens/putida* ensemble of the fluorescent *Pseudomonas* (rRNA homology group I) (Palleroni 1984, 1992). In this rather heterogeneous ensemble all saprophytic (and occasionally animal/human pathogenic) strains were combined that show Arg dihydrolase activity and

do not belong to the well-defined species *P. aeruginosa*. During the last few years a tendency can be observed to break up the *fluorescens/putida* conglomerate by defining new species. In this course *P.* sp. CFML 96.188 according to an intensive phenotypical characterization involving numerical taxonomy studies (Dabboussi *et al.* 1998) was grouped together with several other strains into a phenotypic subcluster of the fluorescent pseudomonads described later as *P. orientalis* sp. nov. (Dabboussi *et al.* 1999). However, a low level (54%) of DNA/DNA hybridization with the *P. orientalis* type-strain, as well as a high $\Delta T m$ value for the DNA hybrid (8 °C), proved that *P.* sp. CFML 96.188 did not really belong to the proposed new species (Dabboussi *et al.* 1999). Inter-

estingly, siderotype studies were in agreement with this conclusion: the CFML 96.188 strain behaved differently from the *P. orientalis* strains, exhibiting a particular PVD-IEF pattern and a specific pyoverdine-mediated iron uptake behavior as compared with the *P. orientalis* strains. The structure elucidation of the respective pyoverdines characterizing these strains was, therefore, of importance.

The second interesting feature of *Pseudomonas* sp. CFML 96.188 is, as shown in this study, that it accepts beside its own pyoverdine six other pyoverdines of foreign origin, though with a somewhat lower rate (Figure 3). They have several structural details in common. The N-terminal sequence D-Ser – basic amino acid (L-Lys, less frequently Orn or Arg) is a very common pattern amongst pyoverdines. Derivatization reactions (e.g., Kinzel *et al.* 1998; Kinzel & Budzikiewicz 1999) suggest that these two amino acids do not play an essential role in the recognition at the cell surface. Moreover, two pyoverdines (n° 3 and 12 in Figure 3) sharing this motive were inefficient in iron uptake. The other common feature is that the C-terminus of the peptide chain forms a cyclopeptidic substructure consisting of three or four amino acids (for *P. veronii* see above) formed by an amide bond between the C-terminal carboxyl group and the ε-amino group of an in-chain L-Lys. Lys is followed by FoOHOrn (D or L). The rest of the cycle is made up of Ser, Thr or Glu (Table 3).

Uptake of heterologous pyoverdines with a cyclopeptidic C-terminus has been noted before (Hohnadel & Meyer 1988). Moreover, crossfeeding studies also showed recognition between strains producing different pyoverdines (Amann *et al.* 2000). From the data presented in Figure 3 it is obvious that cross-recognition is possible only between pyoverdines possessing the C-terminal cyclopeptide substructure. Two of the pyoverdines tested which have a cyclodepsipeptidic C-terminus (lactone instead of lactam formation) are not accepted (n° 8, Wong-Lun-Sang *et al.* 1996, and n° 31, Voßen *et al.* 1999). Admittedly also the amino acid composition of the cycles (*Ser*-Ser OH*Asp*-Thr and Thr-*Ala*-*Ala*-FoOHOrn-Lys, respectively) differs considerably from the examples given in Table 3 where the structures of the pyoverdines with a cyclopeptidic C-terminus are arranged to show structural analogies. The pyoverdines n° 3–5, 6, 9–11, 13–15, 20, 21, 32 and 33 in Figure 3 have a straight peptide chain with a C-terminal cOHOrn residue (n° 6 with a Gly and 33 with an AcOHOrn residue). None of them is taken up

by I. The structure of the pyoverdines not mentioned above is presently unknown.

In all cyclopeptidic pyoverdines the L-Lys residue belonging to the cycle is bound by its α-amino group to the preceding amino acid of the peptide chain, by its carboxyl group to FoOHOrn (preferentially, but not necessarily L) providing one of the binding sites for Fe^{3+}, and by its ε-amino group to L-Ser (in one case L-Thr). This triade of amino acids can, therefore, be assumed to be involved in the binding of the ferri-pyoverdine to the outer membrane receptor. There must be another strain-specific structural feature responsible for the partial acceptance in the cross-uptake experiments. Between the N-terminal unit D-Ser-L-Lys(Arg) and the first FoOHOrn and than again up to the cycle only small neutral amino acids (Gly, Ser) are present. They can be missing on either side of the first FoOHOrn or even on both sides as for I. As the steric structure of the pyoverdines around Fe^{3+} is determined by the three complexing sites (the chromophore and the two FoOHOrn) it may be surmised that the Gly/Ser units are spacers responsible for the general shape of the molecule. It is worth mentioning that the strain producing I which has no spacers on either side of FoOHOrn and which must, therefore, have a very tight structure, accepts all other cyclopeptidic pyoverdines tested. The length of the linear part of the peptide chain should thus be another important structural feature for an efficient pyoverdine/receptor recognition.

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